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Systematics and Species Validity of the Dabieshan Pit Viper *Protobothrops dabieshanensis* Huang *et al.* 2012: Evidence from a Mitochondrial Gene Sequence Analysis

Baowei ZHANG[1*, **], Xin HUANG[1*], Tao PAN[1], Liang ZHANG[2], Wenliang ZHOU[1], Tao SONG[1] and Demin HAN[1]

[1] *School of Life Sciences, Anhui University, Hefei* 230039, *Anhui, China*
[2] *Guangdong Entomological Institute and South China Institute of Endangered Animals, Guangzhou* 510260, *Guangdong, China*

Abstract Aimed to evaluate the phylogenetic position of the recently described *Protobothrops dabieshanensis* Huang *et al.* (2012), phylogenic relationships of 12 species within *Protobothrops* based on four mtDNA gene fragments (12S RNA, 16S RNA, ND4 and Cyt *b*) were reconstructed in our study. The result indicates a clade composed of *P. dabieshanensis*, *P. jerdonii* and *P. xiangchengsis* with strong support. The genetic distance among *P. dabieshanensis*, *P. jerdonii* and *P. xiangchengsis* was much lower than other congeners. Based on the data from the phylogenetic analysis and previously described morphological differences, we conclude that *P. dabieshanensis* is a valid species with close affinities to *P. jerdonii* and *P. xiangchengsis*.

Keywords phylogeny, Crotalinae, systematics, venomous snakes

1. Introduction

Based on osteological features and scale ultrastructure, the genus *Protobothrops* was erected in 1983 (Hoge and Romano-Hoge, 1983; Orlov *et al.*, 2009). Over the past two decades, *Protobothrops* has been systematacially studied on phylogeny and classification (Guo *et al.*, 2006). Presently, the genus *Protobothrops* contains the following 14: *P. elegans*, *P. flavoviridis*, *P. sieversorum*, *P. tokarensis*, *P. trungkhanhensis*, *P. maolanensis*, *P. jerdonii*, *P. xiangchengsis*, *P. cornutus*, *P. kaulbacki*, *P. mangshanensis*, *P. mucrosquamatus*, *P. himalayanus* and *P. dabieshanensis* (Guo *et al.*, 2009; Huang *et al.*, 2012; Liu *et al.*, 2012; McDiarmid *et al.*, 1999; Pan *et al.*, 2013; Zhao *et al.*, 1998). Among the 14 species, the latter nine species are distributed in China (Huang *et al.*, 2012; McDiarmid *et al.*, 1999; Pan *et al.*, 2013; Yang *et al.*, 2011; Zhao *et al.*, 1998). The phylogenetic relationships among most of the species of the genus *Protobothrops* are well established (Yang *et al.*, 2011). Although opinions vary as to the taxonomic validity of this genus (McDiarmid *et al.*, 1999; Zhao *et al.*, 1998), recent molecular phylogenetic studies provided evidence supporting that *Protobothrops* represents a distinct evolutionary lineage (Guo *et al.*, 2007; Guo *et al.*, 2006; Liu *et al.*, 2012).

Recently, a new pit viper was described, *Protobothrops dabieshanensis*, which differs from all other species of *Protobothrops* in morphology and color pattern characteristics (Huang *et al.*, 2012). For a new species, it is important to know its position in phylogeny, at the same time, further molecular information should be provided to support it as a distinct species. The aim of this study is two-fold: 1) to provide a further evidence to support *P. dabieshanensis* as a valid species; 2) to determine the phylogenetic position of *P. dabieshanensis.*

2. Material and Methods

The type specimen (AHU 2011-EE-Hp01) of *P. dabieshanensis* (Huang *et al.*, 2012) and other samples (AHU

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[*] Both authors contributed equally to this work.
[**] Corresponding author: Dr. Baowei ZHANG, from the College of Life Sciences, Anhui University, Hefei, China, with his research focusing on molecular ecology of amphibians.
E-mail: zhangbw@ahu.edu.cn

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2012-EE-Hp01) were all collected from the type locality Yaoluoping Nature Reserve in the Dabie Mountains. Genomic DNA was extracted from 95% ethanol-preserved tissues using standard proteinase K and phenol-chloroform protocols (Hillis *et al.*, 1996). *Cyt*ochrome *b* (Cyt *b*), NADH dehydrogenase subunit 4 (ND4), 12S RNA and 16S RNA sequences of *P. dabieshanensis* were generated using primers L14910/H16064 for Cyt *b* (Burbrink *et al.*, 2000), ND4.light/ Leu3.heavy for ND4 (Arevalo *et al.*, 1994), L1091/ H1557 for 12S RNA (Knight and Mindell, 1993), and L2510/ H3059 for 16S RNA (Guo *et al.*, 2009; Liu *et al.*, 2012). PCR products were purified using commercial kits and double stranded products were sequenced using an ABI 3730 Genetic Analyzer (Applied Biosystems, Foster City, CA, USA), following the manufacturer's protocols. Generated sequences of *P. dabieshanensis* were deposited in GenBank (Accession Numbers: KF003000-KF003004). The existing sequences data of 13 species (20 samples in all) were used from GenBank (Table 1). Among all species of *Protobothrops*, *P. jerdonii* is the most widely distributed and has been studied phylogeographically, so five representative samples of *P. jerdonii* were used, they represented five major lineages of *P. jerdonii* (Guo *et al.*, 2009; 2011). In accordance with previous researches, *Gloydius blomhoffii* and *Ovophis monticola* were chosen as outgroups (Malhotra and Thorpe, 2004; Pyron *et al.*, 2011).

Sequences were aligned by ClustalW (Larkin *et al.*, 2007) in Mega v4.0 (Tamura *et al.*, 2007), with default parameters. Using Bayesian inference (BI), maximum parsimony (MP) and maximum-likelihood (ML) methods, we reconstructed phylogenetic relationships (Posada, 2003). For BI, three partitioning strategies were evaluated: data undivided, four partitions (Cyt *b*, ND4, 12S RNA and 16S RNA) and eight partitions (Cyt *b* and ND4 1st,

Table 1 Specimen details and GenBank accession numbers for the species sampled in this analysis.

Species	Locality	Voucher specimen	GenBank accession No. (Cyt *b*, ND4, 12S, 16S)
Protobothrops mucrosquamatus (1)	Taiwan, China	AM A233	AF171897, AY294265, AY294279, AY294270
P. mucrosquamatus (2)	Vin Phuc, Vietnam	AM B106	AY294275, AY294266, AY294280, AY294271
P. xiangchengensis (1)	Sichuan, China	GP 26/SCUM 035042	DQ666061, DQ666058, AY763188, AY763207
P. xiangchengensis (2)	Sichuan, China	GP 27/SCUM 035046	DQ666062, DQ666059, AY763189, AY763208
P. cornutus	Vietnam	ZFMK 75067	AY294272, AY294262, AY294276, AY294267
P. elegans	Ryukyu Is., Japan	UMMZ 199970	AY223575, U41893, AF057201, AF057248
P. flavoviridis	Ryukyu Is., Japan	UMMZ 199973	AY223574, U41894, AF057200, AF057247
P. tokarensis	Ryukyu Is., Japan	FK 1997	AY223576, AY223628, AF057202, AF057249
P. kaulbacki	Xizang, China	GP 112/SYNU 0400II30	DQ666060, DQ666057, DQ666056, DQ666055
P. sieversorum	Vietnam	AM B162	AY352753, AY352816, AY352782, AY352721
P. mangshanensis	Hunan, China	AM B300	AY352758, AY352821, AY352787, AY352726
P. maolanensis (1)	Guizhou, China	GP 1883/YBU 11157	JN799401, JN799409, JN799405, JN799397
P. maolanensis (2)	Guizhou, China	GP 1884/YBU 11158	JN799402, JN799410, JN799406, JN799398
*P. jerdon*i (A)	China	AM B596	EU809987, EU810007, EU809954, EU809971
P. jerdonii (B)	China	AM B485	AY294273, AY294263, AY294277, AY294268
P. jerdonii (C)	China	GP345	HM567484, HM567416, -, -
*P. jerdon*ii (D)	Nujiang, China	CAS 215051	AY294274, AY294264, AY294278, AY294269
P. jerdonii (E)	Zayu, Xizang	GP 587	HM567500, HM567432, -, -
P. dabieshanensis (1)	Anhui, China	AHU 2011-EE-Hp01	KF003002, KF003003, KF003000, KF003001
P. dabieshanensis (2)	Anhui, China	AHU 2012-EE-Hp01	KF003004, KF003004, KF003004, KF003004
Ovophis monticola	China	AM B482	AY352748, AY352809, AY352775, AY352714
Gloydius blomhoffi	Teuri Isl., Japan	AM B524	AY352751, AY352814, AY352780, AY352719

Abbreviations: CAS: California Academy of Science, San Francisco, USA; SCUM: Sichuan University Museum, China; SYNU: Shenyang Normal University, China; UMMZ: University of Michigan Museum of Zoology; ZFMK: Zoologisches Forschungsinstitut und Museum Alexander Koenig, Bonn, Germany; YBU: Yibin University, China; AHU-EE: Anhui University, Evolution and Ecology group; AM: Anita Malhotra, own catalogue number; FK: Fred Kraus, field tag; GP: Guo Peng, own catalogue number; - : without the corresponding sequence information.

2nd and 3rd positions separately, 12S RNA and 16S RNA). Each partition was assigned best-fit substitution model using *AIC* in MrModeltest 2.3 (Nylander, 2004). The optional partition model was chosen using Bayes factors (BF) (Brown and Lemmon, 2007). BI analysis was performed using MrBayes 3.1.2 (Huelsenbeck and Ronquist, 2001; Ronquist and Huelsenbeck, 2003), with three runs each with four Markov chains (three heated chains and a single cold chain) using a random starting tree. Each run was conducted with a total of 5×10^6 generations and sampled every 1000 generations; we discarded the first 1.5×10^6 generations as burn-in after evaluation in the program Tracer 1.4. Trees from each run were combined to calculate posterior probabilities (PPs). MP trees were obtained using PAUP v4.0b10 (Swofford, 2003) with a heuristic search using 1000 random sequence addition replicates and tree bisection-reconnection (TBR) branch swapping. Support values (bootstrap-BS) were calculated using 1000 pseudo replicates (Felsenstein, 1985). The ML analysis was conducted with PAUP v4.0b10 (Swofford, 2003). Support for the ML phylogenetic reconstruction was assessed using bootstrap resampling with 1000 pseudoreplicates. Average divergence estimates (1. P-distance for DNA sequences; 2. Composition distance for amino acid sequences) between species were calculated from sequences as well as from each marker separately in Mega v4.0 (Tamura *et al.*, 2007).

3. Results

A total of 2041 base pairs (bp) were obtained (625 bp of Cyt *b*, 575 bp of ND4, 381 bp of 12S RNA and 460 bp of 16S RNA) in two samples of *P. dabieshanensis*. Of these 2041 bp, 308 base pairs are parsimony informative. There were no deletions, insertions or stop codons in the two protein-coding genes (Cyt *b* and ND4), so no evidence of the unintentional amplification of nuclear pseudogenes was detected. The uncorrected mean interspecific sequence divergence ranges from 0.8% (*P. flavoviridis vs. P. tokarensis*) to 8.1% (*P. mucrosquamatus vs. P. sieversorum*) for 12S RNA, 1.3% (*P. xiangchengensis vs. P. jerdonii*) to 7.9% (*P. tokarensis vs. P. sieversorum*) for 16S RNA, 2.6% (*P. flavoviridis vs. P. tokarensis*) to 13.6% (*P. sieversorum vs. P. elegans*) for ND4, 1.8% (*P. flavoviridis vs. P. tokarensis*) to 16.5% (*P. mucrosquamatus vs. P. sieversorum*) for Cyt *b* and 2.3% (*P. flavoviridis vs. P. tokarensis*) to 11.6% (*P. sieversorum vs. P. elegans*) for the concatenated data. To compare *P. dabieshanensis* with other species of *Protobothrops*, the mean interspecific sequence divergence ranges from 2.9% (*P. dabieshanensis vs. P. jerdonii*) to 6.3% (*P. dabieshanensis vs. P. sieversorum*) for 12S RNA, 1.5% (*P. dabieshanensis vs. P. mangshanensis*) to 5.5% (*P. dabieshanensis vs. P. sieversorum*) for 16S RNA, 5.4% (*P. dabieshanensis vs. P. xiangchengensis*) to 12.3% (*P. dabieshanensis vs. P. kaulbacki*) for ND4, 7.0% (*P. dabieshanensis vs. P. jerdonii*) to 14.9% (*P. dabieshanensis vs. P. sieversorum*) for Cyt *b*, 5.0% (*P. dabieshanensis vs. P. jerdonii*) to 10.5% (*P. dabieshanensis vs. P. sieversorum*) for the concatenated data (Table 2). Distances among 12 *Protobothrops* species of concatenated DNA sequences and amino acid sequences (Cyt *b* and ND4) have showed in spatial distribution patterns (Figure 1). In the spatial distribution patterns, the dots representing the distances of *P. dabieshanensis* to other species mixed with these of other distances (Figure 1), and it supported that *P. dabieshanensis* should be looked as distinct species as well as other species in *Protobothrops*.

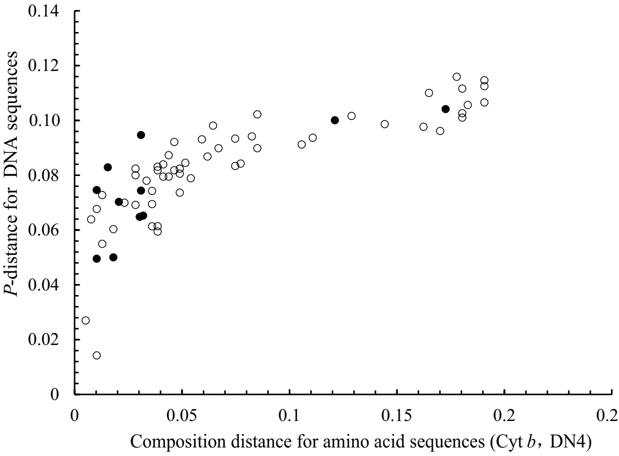

Figure 1 The spatial distribution patterns of 12 species in *Protobothrops*, based on p-distance and composition distance. Solid circles: interspecific sequences divergence relate to *P. dabieshanensis*; open circles: interspecific sequences divergence among congeneric species, except *P. dabieshanens*.

From the phylogenetic analyses, BI, ML, and MP trees all produced same topologies (Figure 2), which were consistent with previous studies (e.g. Guo *et al.*, 2009; Liu *et al.*, 2012; Malhotra and Thorpe, 2004; Yang *et al.*, 2011). Twelve species of *Protobothrops* form a distinct monophyletic lineage with *P. kaulbacki* (distributed in China and Myanmar) and *P. sieversorum* (Laos, Vietnam) comprises a basal sister clade. In the subclade, *P. mangshanensis* is sister to the remaining species. Furthermore, the remaining 9 species converged into 2 subclades. The first clade is comprised of *P.*

Table 2 Mean interspecific genetic divergence estimates (‰, *P*-distances). Above diagonal: distances calculated from 12S RNA/16S RNA; below diagonal, distances calculated from ND4 / Cyt *b* / 12S+16S+ND4+Cyt *b*. Abbreviations: P. mu: *P. mucrosquamatus*; P. je: *P. jerdonii*, P. co: *P. cornutus*, P. fl: *P. flavoviridis*, P. el: *P. elegans*, P. to: *P. tokarensis*, P. si: *P. sieversorum*, P. ka: *P. kaulbacki*, P. xi: *P. xiangchengensis*, P. ma: *P. mangshanensis*, P. ml: *P. maolanensis*, P. da: *P. dabieshanens.*

	P. mu	P. xi	P. co	P. el	P. fl	P. to	P. ka	P. si	P. ma	P. ml	P. je	P. da
P. mu		71/24	55/22	39/24	52/28	47/44	66/52	81/50	69/26	37/17	63/28	58/28
P. xi	94/107/78		50/22	63/26	50/31	50/46	58/48	68/55	40/20	61/28	31/13	39/17
P. co	96/138/84	68/91/61		55/35	45/22	45/39	61/57	66/59	53/24	47/24	39/31	34/22
P. el	80/106/68	106/130/87	106/136/90		58/39	52/55	60/52	76/63	64/28	34/24	55/35	55/31
P. fl	108/120/83	82/98/69	90/114/74	106/136/91		8/24	52/57	52/61	51/33	45/37	37/31	37/35
P. to	102/122/85	79/101/73	84/109/74	100/141/93	26/18/23		52/76	52/79	51/48	39/52	37/46	37/50
P. ka	113/131/96	106/138/94	111/150/102	127/138/101	118/138/98	116/139/102		47/57	61/46	66/57	50/52	52/48
P. si	125/165/112	117/159/107	129/164/112	136/161/116	130/162/110	131/164/115	111/120/90		66/53	76/50	60/59	63/55
P. ma	124/125/92	98/98/69	99/116/79	122/132/93	103/117/82	96/114/82	94/117/84	125/124/98		53/22	40/24	45/15
P. ml	77/74/55	106/106/80	104/120/81	85/83/61	101/112/79	96/120/83	127/123/99	125/146/106	110/117/82		53/37	45/24
P. je	104/114/82	33/40/27	66/86/59	103/130/87	75/83/60	72/86/64	120/128/94	115/149/103	99/95/70	106/102/79		29/24
P. da	97/125/84	54/75/51	89/93/65	122/141/95	80/91/65	82/94/70	123/146/100	122/149/105	108/105/74	101/106/75	63/70/50	

mucrosquamatus, *P. elegans* and *P. maolanensis* with stronge support. The other clade contains 6 species, *P. xiangchengsis*, *P. jerdonii*, *P. dabieshanensis*, *P. cornutus*, *P. flavoviridis* and *P. tokarensis*, with strong support as well. Within this clade, *P. flavoviridis* and *P. tokarensis* composed a distinct sister branch as to *P. xiangchengsis*, *P. jerdonii*, *P. dabieshanensis* and *P. cornutus*. Within the latter 4 species, *P. xiangchengensis* showed a very close relationship to *P. jerdonii*, they were sister group of *P. dabieshanensis* with considerable supports. Subsequently, they joined with *P. cornutus* in the phylogenetic tree. Evidently, *P. dabieshanensis* comprised a distinct phylogenetic lineage within *Protobothrops*, with a close relationship to *P. xiangchengensis* and *P. jerdonii*.

4. Discussion

In the past 13 years, 16 new species of snakes were discovered in China. Within *Protobothrops*, besides *P. dabieshanensis*, three new species (*P. trungkhanhensis*, *P. maolanensis* and *P. himalayanus*) had been found not long ago (Huang *et al.*, 2012; Orlov *et al.*, 2009; Yang *et al.*, 2011). For these new species, it is important to explore their phylogenetic relationships. At present, a molecular systematic approach is used to address this question. In our present study, the uncorrected mean interspecific sequence divergence of *Protobothrops* ranges from 0.8% to 8.1% for 12S RNA, 1.3% to 7.9% for 16S RNA, 2.6% to 13.6% for ND4, 1.8 % to 16.5% for Cyt *b* and 2.3% to 11.6% for the concatenated data (Table 2). The ranges of genetic divergences of *P. dabieshanensis* with other species were interspecific rather than intraspecific, whatever in concatenated gene sequences or amino acid sequences (Table 2, Figure 1). On the other hand, according to the phylogeny, *P. dabieshanensis* is an evolutionarily independent lineage and is thus strongly supported as a separate species with close relationships to *P. jerdonii* and *P. xiangchengensis*. As the same time, there are significant differences between *P. dabieshanensis, P. jerdonii* and *P. xiangchengensis* in morphology and color pattern characters (Huang *et al.*, 2012). The analysis supported the definite phylogenetic relationships among 12 of 13 defined congeneric species, except *P. trungkhanhensis*. However, there are obvious external differences in appearance between *P. dabieshanensis* and *P. trungkhanhensis* (Yang *et al.*, 2011). On the other hand, *P. trungkhanhensis* is found in Vietnam (Liu *et al.*, 2012), and *P. dabieshanensis* is found in the Dabie Mountains (Huang *et al.*, 2012). There are far away geographic locations between them, so we can exclude the possibility

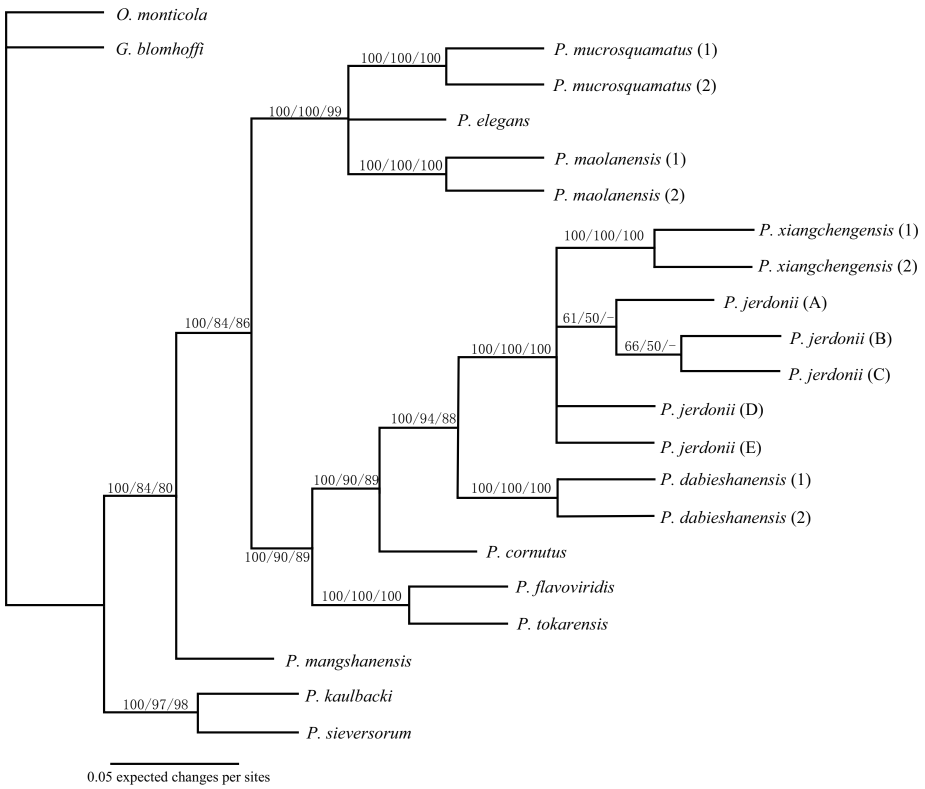

Figure 2 The Bayesian 50% majority-rule consensus tree of *Protobothrops* inferred from the combined dataset of 12S RNA, 16S RNA, Cyt *b* and ND4 based on a four-partition strategy. Posterior probabilities and bootstrap values (BI/ML/MP) for the clades are shown in this order. Support values < 50% are indicated by "-".

they are the same species. So, it is safe to declare that *P. dabieshanensis* and *P. trungkhanhensis* are different species of *Protobothrops*. Based on the above discussions and analyses, we think that *P. dabieshanensis* is a valid species in *Protobothrops*. Whereas, above conclusion just based on mitochondrial markers sequence analyses, more genetic markers (e.g. multi-locus nuclear gene) data should be employed in the further study to provide more and strong evidences for its species delimitation.

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